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学位論文題目	Anti-austerity activities of Japanese Kampo medicines and active constituents of *Andrographis paniculata* （漢方生薬の栄養飢餓状態選択的細胞毒性と穿心蓮の活性成分）

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論文内容の要旨

Cancer is a major public health problem throughout the world. Among the different forms of cancer, pancreatic cancer is most aggressive and has an exceptionally high global mortality rate which ranks 7th as the most frequent cause of cancer death. Pancreatic cancer rapidly metastases and leads to the death of patients in a short period after diagnosis. Thus, the 5-year survival rate of patients with pancreatic cancer is the lowest among several cancers. Though surgery is the only treatment that offers any prospect of potential cure, chemotherapy with 5-fluorouracil and gemcitabine is also used for palliative therapy of advanced pancreatic cancer. However, pancreatic cancer is largely resistant to most known chemotherapeutic agents, including 5-fluorouracil and gemcitabine. Therefore, effective chemotherapeutic agents for pancreatic cancer are urgently needed.

Tumor cells, in general, proliferate very fast, and demand high essential nutrients and oxygen. The immediate environment of cancers increasing in size, however, often becomes heterogeneous and some regions of large cancers often possess micro environmental niches, which exhibit a significant gradient of critical metabolites, including oxygen, glucose, other nutrients, and growth factors. Thus, many cancer cells obtain their critical metabolites by randomly recruiting new blood vessels, a phenomenon commonly known as angiogenesis, to survive under such severe conditions. However, human pancreatic cancer survives with an extremely poor blood supply and becomes more malignant. The method by which pancreatic cancer survives is by obtaining a remarkable tolerance to extreme nutrient starvation. Therefore, it has been hypothesized that eliminating the tolerance of cancer cells to nutrition starvation is known as “anti-austerity” strategy for cancer therapy.

In this study, on the discovery of anticancer agents from natural products, I first examined preferential cytotoxicity of crude drugs available in Japan against human pancreatic cancer cell lines PANC-1 and PSN-1 based on anti-austerity strategy. Among the tested crude drugs, the extract of Andrographis Herba (No.17, aerial part of *Andrographis paniculata*) showed the most potent preferential cytotoxicity against PANC-1 and PSN-1cells. Hence, I investigated active constituents of *A. paniculata* and identified them for the preferential cytotoxicity against PANC-1 and PSN-1 cells. Furthermore, I examined detailed mechanism of the preferential cytotoxicity of the active constituent.

1. Preferential cytotoxicity of crude drugs available in Japan against human pancreatic cancer cell lines

The 70% EtOH extracts of 24 crude drugs available in Japan were screened for their preferential cytotoxicity against PANC-1 and PSN-1 cells. The preferential cytotoxicity was determined by comparing the cytotoxicities in normal medium

(DMEM) and nutrient deprived medium (NDM). Of the 24 extracts examined, nine extracts of Isodonis Herba (extract No.1, aerial part of *Plectranthus japonicus*), Phellodendri Cortex (No.2, bark of *Phellodendron amurense*), Lacca Sinica Exsiccata (No.3, resin of *Rhus verniciflua*), Arctii Fructus (No.5, fruits of *Arctium lappa*), Lycopodium (No.15, spore of *Lycopodium clavatum*), Agrimoniae Herba (No.16, aerial part of *Agrimonia pilosa*), Andrographis Herba (No.17, aerial part of *Andrographis paniculata*), Panacis Japonici Rhizoma (No.18, rhizome of *Panax japonicus*), and Chorei (No.19, sclerotium of *Polyporus umbellatus*) induced cell death to both cell lines in NDM, but not in normal medium (DMEM) (Table 1). Among them, Andrographis Herba (No.17) exhibited the most potent preferential cytotoxicity against PANC-1 and PSN-1 cells with the PC_{50} values of 9.72 μg/mL and 9.41 μg/mL, respectively. Isodonis Herba (No.1), Phellodendri Cortex (No.2), Lycopodium (No.15), and Panacis Japonici Rhizoma (No.18) showed also mild activity. The ethidium bromide (EB)/acridine orange (AO) double-staining on PANC-1 and PSN-1 cells treated by these extracts predicted that Isodonis Herba Herba (No.1), Lycopodium (No.15), and Panacis Japonici Rhizoma (No.18) induced the apoptosis-like cell death under nutrient starvation whereas Phellodendri Cortex (No.2) and Andrographis Herba (No.17) induced necrosis-like cell death.

2. Preferentially cytotoxic constituents of *Andrographis paniculata*

To further investigate active constituents against PANC-1 and PSN-1 cells, the most potent crude drug, Andrographis Herba (No.17) was selected, and bioassay-guided isolation of constituents was carried out. The 70% EtOH extract was subjected to a series of chromatographic separation which led to the isolation of 20 compounds: stearic acid (**1**), pinostrobin (**2**), β-sitosterol (**3**), 5-hydroxy-6,7-dimethoxyflavone (**4**), pinocembrin (**5**), ermanin (**6**), ergosterol peroxide (**7**), oleanolic acid (**8**), 5,3′,4′-trihydroxy-7-methoxyflavone (**9**), andrograpanin (**10**), skullcapflavone I (**11**), loliolide (**12**), 5-hydroxy-7,8,2′,5′-tetramethoxyflavone (**13**), 3-oxo-*ent*-cleroda-8(17),11,13-rtien- 16,15-olide (**14**), 5,7,8-trimethoxyflavanone (**15**), 14-deoxy-11,12-didehydro- andrographolide (**16**), isoandrographolide (**17**), andrographolide (**18**), apigenin (**19**), and 14-deoxyandrographolide (**20**) (Figure 1). Among these compounds, **2** and **5** have been isolated for the first time from the plants of *Andrographis* genus. Of the 20 compounds examined, **16** exhibited the most potent activity against PANC-1 and PSN-1 cells with the PC_{50} values of 10.0 μM and 9.27 μM, respectively. The six compounds, **7**, **8**, **10**, **12**, **13**, and **18** showed also the preferential cytotoxicity with mild potency (PC_{50}: 34–85 μM) (Table 2).

3. Preferential cytotoxicity of 14-deoxy-11,12-didehydroandrographolide (16)

In a continuing study, the nutrient-dependency of the preferential cytotoxicity of 14-deoxy-11,12-didehydroandrographolide (**16**), which showed the most potent activity, was examined. As a result, **16** inhibited survival of PANC-1 cells under deprivation of amino acids or serum, whereas **16** caused cell death of PSN-1 under

deprivation of serum (Figure 2). On the other hand, the mechanism of cell death induced by **16** was examined using microscopical observation, EB/AO double staining, and flow cytometry with propidium iodide/annexin V double staining. Interestingly, microscopic observation of the PANC-1 and PSN-1 cells treated by **16** displayed the membrane bleb, nuclear fragmentation, and chromatin condensation, which are typical apoptosis-like morphological changes. Further EB/AO double-staining experiment in the cells treated with **16** under nutrient starvation indicated that condensed and/or fragmented chromatin is stained in orange, which allowed us to predict that **16** induces apoptosis-like cell death (Figure 3). Finally, flow cytometry with propidium iodide/annexin V double staining of **16** against PANC-1 and PSN-1 cells in NDM indicated that it also triggered apoptosis-like cell death in a concentration- and time-dependent manner. These results suggested that **16** induced apoptosis-like cell death to PANC-1 and PSN-1 cells under nutrient starvation.

Conclusions

In a course of search for anticancer agent based on a novel anti-austerity strategy, I found that 70% EtOH extracts of the crude drug extracts of Isodonis Herba (No.1), Phellodendri Cortex (No.2), Lycopodium (No.15), Andrographis Herba (No.17), and Panacis Japonici Rhizoma (No.18) showed the preferential cytotoxicity against PANC-1 and PSN-1 cells. Among them, Andrographis Herba (No.17) exhibited the most potent preferential cytotoxicity against PANC-1 and PSN-1 cells. Phytochemical investigation of this active extract led to the isolation of 20 compounds consisting of six labdane-type diterpenes (**10**, **14**, **16**–**18**, **20**), six flavones (**4**, **6**, **9**, **11**, **13**, **19**), three flavanones (**2**, **5**, **15**), two sterols (**3**, **7**), a fatty acid (**1**), a triterpene (**8**), and a monoterpene (**12**). Among these, 14-deoxy-11,12-didehydroandrographolide (**16**) displayed the most potent preferential cytotoxicity against PANC-1 and PSN-1 cells with PC_{50} value of 10.0 μM and 9.27 μM, respectively. Microscopical observation, EB/AO double staining, and flow cytometry with propidium iodide/annexin V double staining predicted that **16** triggered apoptosis-like cell death in NDM with an amino acids and/or serum-sensitive mode. These results suggest that the Andrographis Herba (aerial part of *A. paniculata*) and its active constituent, 14-deoxy-11,12-didehydro-andrographolide (**16**) may be key leads in the development of new drugs based on the anti-austerity strategy.

Reference

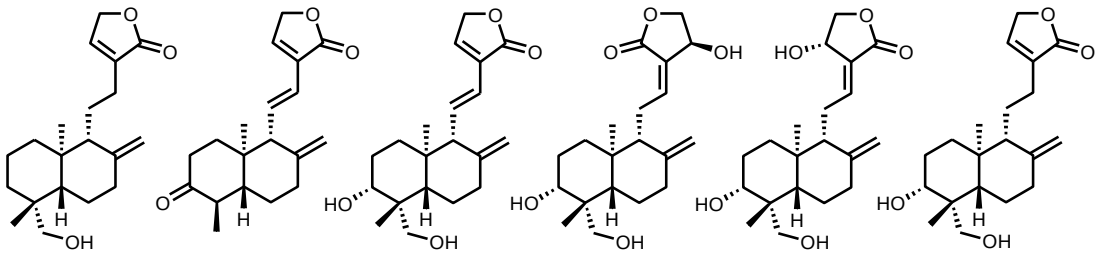
1. Lee, S.; Dibwe, D. F.; Li, F.; Morita, H.; Tezuka, Y., Preferential cytotoxicity of crude drugs used in Japanese Kampo medicines against human pancreatic cancer PANC-1 and PSN-1 cells, *Traditional & Kampo Medicine*, **2015**, DOI: 10.1002/tkm2.1016.

2. Lee, S.; Morita, H.; Tezuka, Y., Preferential cytotoxic constituents of *Andrographis paniculata* and their preferential cytotoxicity against human pancreatic cancer cell lines, *Natural Product Communications*, **2015**, *in press.*

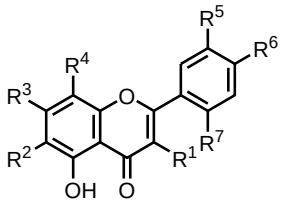
Table 1. Preferential cytotoxicity of crude drugs used in Japanese Kampo medicines against human pancreatic cancer cell lines PANC-1 and PSN-1.

Extract No.	PC_{50} (μg/mL)[a]		Extract No.	PC_{50} (μg/mL)[a]	
	PANC-1	PSN-1		PANC-1	PSN-1
1	35.1	37.1	17	9.72	9.41
2	38.1	41.6	18	37.0	36.6
3	80.5	83.6	19	69.0	54.2
5	71.7	78.1	Others	>100	>100
7	97.1	> 100	Arctigenin[b]	0.29	0.47
15	36.3	17.9	Paclitaxel[c]	>100	>100
16	59.2	91.0			

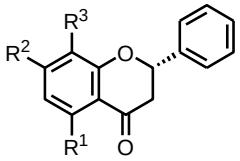
[a] Preferentially 50% growth inhibitory concentration in NDM.
[b,c] Positive and negative control, respectively.



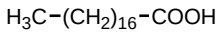
10 **14** **16** **17** **18** **20**



4 $R^1 = R^4 = R^5 = R^6 = R^7 = H$, $R^2 = R^3 = OMe$
6 $R^1 = R^6 = OMe$, $R^2 = R^4 = R^5 = R^7 = H$, $R^3 = OH$
9 $R^1 = R^2 = R^4 = R^7 = H$, $R^3 = OMe$, $R^5 = R^6 = OH$
11 $R^1 = R^2 = R^5 = R^6 = H$, $R^3 = R^4 = OMe$, $R^7 = OH$
13 $R^1 = R^2 = R^6 = H$, $R^3 = R^4 = R^5 = R^7 = OMe$
19 $R^1 = R^2 = R^4 = R^5 = R^7 = H$, $R^3 = R^7 = H$



2 $R^1 = OH$, $R^2 = OMe$, $R^3 = H$
5 $R^1 = R^2 = OH$, $R^3 = H$
15 $R^1 = R^2 = R^3 = OMe$



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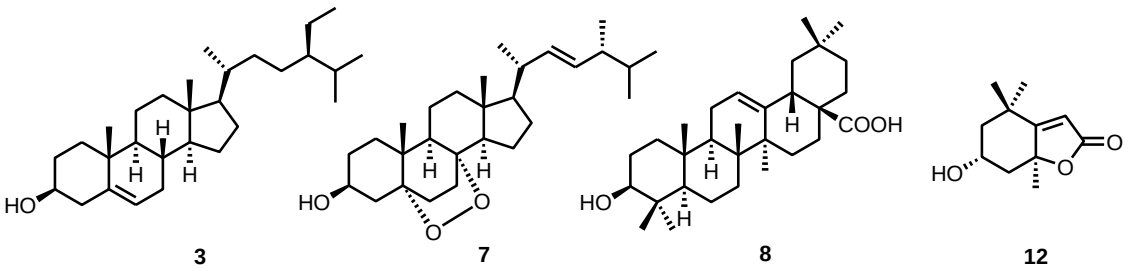


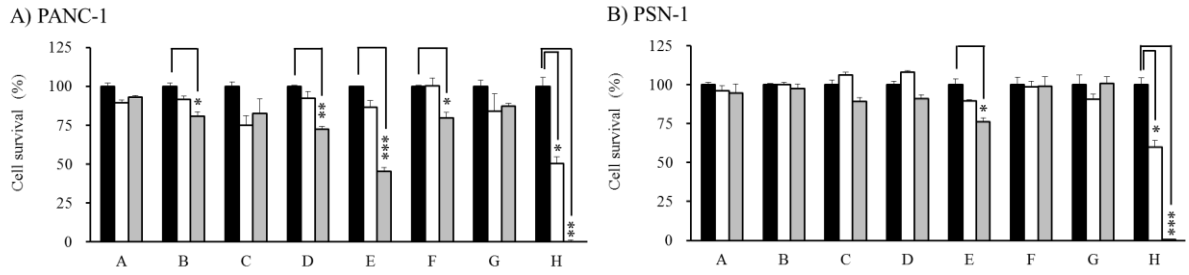
3 **7** **8** **12**

Figure 1. Structures of compounds **1**–**20**.

Table 2. Preferential cytotoxicity of compounds **1**–**20** on human pancreatic cancer lines PANC-1 and PSN-1.

Compound	PC_{50} (μM)[a]		Compound	PC_{50} (μM)[a]	
	PANC-1	PSN-1		PANC-1	PSN-1
7	60.5	79.2	**16**	10.0	9.27
8	40.4	58.6	**18**	34.3	48.3
10	46.0	43.9	Others	>100	>100
12	85.0	>100	Arctigenin[b]	0.48	0.68
13	74.9	74.1	Paclitaxel[c]	>100	>100

[a] Preferentially 50% growth inhibitory concentration in NDM.
[b,c] Positive and negative control, respectively.



Nutrients	A	B	C	D	E	F	G	H
Glucose	+	+	−	−	+	+	−	−
Amino acid	+	−	+	−	+	−	+	−
Serum	+	+	+	+	−	−	−	−

Figure 2. Nutrient-dependency of preferential cytotoxicity of **16** against human pancreatic cancer cell lines PANC-1 (A) and PSN-1 (B). Compound **16** was added to the medium, and cell survival was examined at 24 h after the start of nutrient deprivation. Data are expressed by the mean values of triplicate determinations ± SD. *** $P < 0.0001$, ** $P < 0.001$, * $P < 0.01$ compared with control.

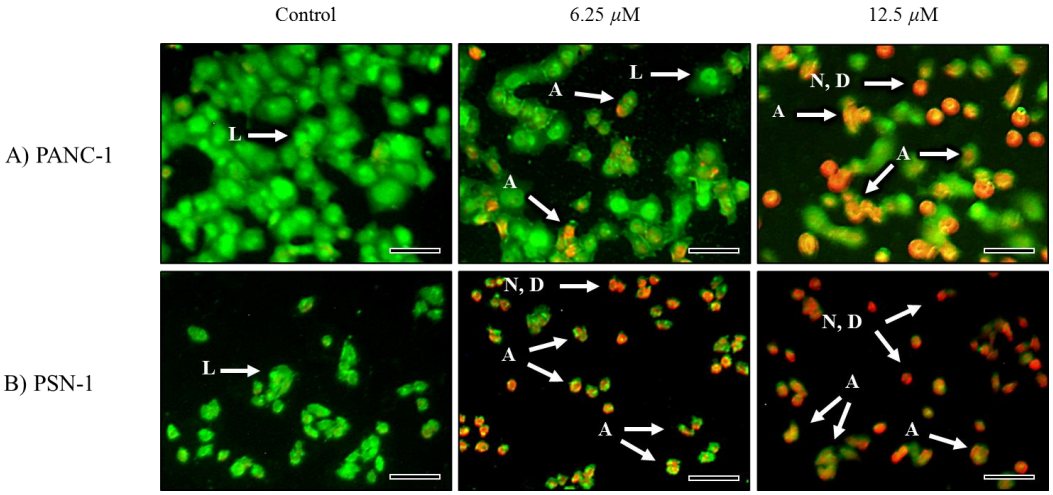


Figure 3. EB/AO staining of PANC-1 (A) and PSN-1 (B) cells treated with **16** in NDM for 12 h. L: live cell, A: apoptotic-like cell, N: necrotic-like cell, D: dead cell.

学 位 論 文 審 査 の 要 旨

膵臓がんは，様々ながんの中で最も進行性で，５年間生存率が５％以下の極めて攻撃的ながんである．その特徴の一つとして，周辺に血管が少なく血液供給の乏しい環境，即ち，栄養供給の少ない状態（栄養飢餓状態）においても生存・増殖できることが挙げられる．興味深いことに，このような栄養飢餓状態では，既存の抗がん剤は膵臓がん細胞に対して細胞毒性を示さないことが報告されている．そのため，栄養飢餓状態の膵臓がん細胞に選択的に細胞毒性を示す漢方方剤や生薬及び阻害物質を見いだすことができれば，あらたな膵臓がん治療薬の開発に結実することが期待される．申請者は，日本で使用される２４種の生薬について，ヒト膵がん細胞PANC-1及びPSN-1に対する栄養飢餓状態での選択的細胞毒性を精査することにより，穿心蓮（*Andrographis paniculata*の地上部）に膵臓がん治療薬としての可能性を見いだした．さらに，穿心蓮の化学成分について，ヒト膵がん細胞に対する栄養飢餓状態での選択的細胞毒性に基づいて単離を進めることにより，あらたな膵臓がん治療薬シードを与えるに至った．本研究の内容の骨子と審査結果は下記に示すとおりである．

1．日本で使用される生薬のヒト膵がん細胞に対する栄養飢餓状態での選択的細胞毒性

先行研究において，ヒト膵がん細胞PANC-1に対して栄養飢餓状態選択的細胞毒性を示していた日本の生薬２４種の70%エタノール抽出エキスについて，PANC-1に対する栄養飢餓状態選択的細胞毒性の再評価及びあらたにヒト膵臓がん細胞PSN-1に対する栄養飢餓状態選択的細胞毒性を評価した．これにより，この内の９種類の生薬（延命草，黄柏，乾漆，牛蒡子，石松子，仙鶴草，穿心蓮，竹節人参，猪苓）がPANC-1とPSN-1に対して有意な栄養飢餓状態選択的細胞毒性を有することを明らかにした．また，その中でも穿心蓮がPANC-1とPSN-1に対して最も強い選択的細胞毒性を示すことを見いだした（PC_{50}はそれぞれ9.72 μg/mL, 9.41 μg/mL）．さらに，強い細胞毒性を示した上位５種類の生薬（延命草、黄柏、石松子、穿心蓮、竹節人参）について各種栄養状態におけるPANC-1とPSN-1に対する細胞毒性を精査することにより，これら５種のPANC-1に対する細胞毒性は全て血清欠乏選択的である一方で，PSN-1に対しては，黄柏は血清とグルコース欠乏選択的に，穿心蓮と竹節人参はアミノ酸と血清欠乏状態選択的に細胞毒性を示すことを明らかにした．蛍光色素染色法を用いて作用メカニズムの検討を行ったところ，延命草，石松子，竹節人参はアポトーシス様の作用により，黄柏と穿心蓮はネクローシス様の作用により両細胞の細胞死を誘導することが明らかとなった．

2. 穿心蓮のヒト膵がん細胞栄養飢餓状態選択的細胞毒性成分の解析

穿心蓮の70%エタノール抽出エキスより，PANC-1に対する栄養飢餓状態選択的細胞毒性を指標に活性成分の単離・精製を進めることで，６種の既知ラブダン型ジテルペン，９種の既知フラボノイド，５種の他骨格を有する既知化合物を単離し，各種スペクトル分析に基づいて構造を決定した．得られた全ての化合物についてPANC-1とPSN-1に対する細胞毒性試験を実施することにより，これらの中で既知ラブダン型ジテルペン14-deoxy-11,12-didehydroandrographolideがPANC-1とPSN-1に対して最も強い栄養飢餓状態選択的細胞毒性を示すことを明らかにした（PC_{50}はそれぞれ10.0 μM, 9.27 μM）．

3. 14-Deoxy-11,12-didehydroandrographolideの栄養飢餓状態選択的細胞毒性の解析

穿心蓮より得た14-deoxy-11,12-didehydroandrographolideの各種栄養状態におけるPANC-1とPSN-1に対する細胞毒性を精査することにより，その細胞毒性が，PANC-1に対してはアミノ酸もしくは血清欠乏選択的である一方で, PSN-1に対しては血清欠乏状態にのみ選択的であることを明らかにした．さらに，顕微鏡による細胞の形態観察と蛍光色素染色法及びフローサイトメトリーを用いることにより，本化合物がアポトーシス様の作用によりPANC-1とPSN-1の細胞死を誘導することを明らかにした．

以上のように，申請者は日本で使用される生薬の穿心蓮がヒト膵がん細胞 PANC-1 及び PSN-1 に対して栄養飢餓状態選択的細胞毒性を示すことを明らかにした．さらに，穿心蓮から２０種の化合物を単離・同定し，そのうちの１種のラブダン型ジテルペンが PANC-1 と PSN-1 に対して比較的強い栄養飢餓状態選択的細胞毒性を示すことを明らかにした．これらの結果は，穿心蓮の膵がんに対する治療薬としての利用，並びに，膵がんに対する新規抗癌剤の開発にあらたな科学的知見を与えたと言える．

主査及び副査は，論文内容と面接試験より，申請者　李雪林に，博士（薬科学）を授けるに値するものと判定した．

1. **Lee, S.**; Dibwe, D. F.; Li, F.; Morita, H.; Tezuka, Y.
Preferential cytotoxicity of crude drugs used in Japanese Kampo medicines against human pancreatic cancer PANC-1 and PSN-1 cells", *Traditional & Kampo Medicine,*
DOI: 10.1002/tkm2.1016

2. **Lee, S.**; Morita, H.; Tezuka, Y.
Preferential cytotoxic constituents of *Andrographis paniculata* and their preferential cytotoxicity against human pancreatic cancer cell lines", *Natural Product Communication*
In press（印刷中）